J Clin Pathol 1987;**40**:1240–1246

Adaptation of methods for glutamate dehydrogenase and alcohol dehydrogenase activities to a centrifugal analyser: assessment of their clinical use in anoxic states of the liver

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SUMMARY Sensitive, precise, and rapid methods for the measurement of alcohol dehydrogenase (ADH) and glutamate dehydrogenase (GDH) were developed on the Cobas Bio centrifugal analyser. The optimal pH for ADH in caucasians was 9·8. Non-linearity of ADH enzyme activity was observed when samples were diluted in saline; linearity was restored when inactivated serum was used as diluent. ADH was shown to be a sensitive index of liver anoxia due to cardiorespiratory disturbance (clinical sensitivity 90%) and generalised anoxia. GDH exhibited sensitivity equal to that of alanine aminotransferase (ALT) but was inferior to gamma-glutamyltransferase (GGT) in the detection of specific liver disease. Both ADH and GDH were sensitive indicators of alcoholic liver disease.

Alcohol dehydrogenase (ADH, EC 1.1.1.1) is a cytoplasmic liver enzyme located predominantly in the centrilobular region of the liver (zone 3 of the hepatic acinus); glutamate dehydrogenase (GDH, EC 1.4.1.2) is predominantly a liver enzyme found in mitochondria. Until recently critical assessment of the diagnostic potential of these two enzymes was limited by (i) their very low activity in normal human serum and (ii) the lack of sensitive spectrophotometric assay techniques to measure their activities.

Several recent reports document differing and often conflicting clinical uses for these enzymes. Fujisawa *et al*[1] stated that ADH is a sensitive marker for chronic hepatitis and chronic aggressive hepatitis, but not for obstructive jaundice. Khayrollah *et al*[2] concluded that ADH is a sensitive indicator for assessing hepatotoxicity due to drugs, hepatocellular damage, infection, inflammation and malignancy. More recently, Kato *et al*[3 4] stated that ADH activity may be raised in patients with hepatic centrilobular damage. Glutamate dehydrogenase was shown by Jung *et al*[5] to be of similar diagnostic sensitivity to mitochondrial aspartate aminotransferase (AST, EC 2.6.1.1) in chronic pathological liver states. Schellenberg *et al*[6] stated that the place of GDH in the laboratory was in the detection of heavy drinkers. Levy[7] concluded that GDH was useful as a cancer marker in hepatic metastases, while Kim *et al*[8] showed that, although GDH was as sensitive and specific as serum alkaline phosphatase activity (AP, EC 3.1.3.1) in the detection of hepatic metastases, it was inferior to gamma-glutamyltransferase (GGT, EC 2.3.2.2) and 5′-nucleotidase (5′NT, EC 3.1.3.5).

This paper describes the development of sensitive routine methods for the measurement of GDH and ADH using a centrifugal analyser. For GDH, a manual kit procedure was adapted for use with the centrifugal analyser. A method for ADH using centrifugal analysis has recently been reported by Kato[3] in Japan. As racial differences in ADH activity, caused by isoenzyme heterogeneity have been documented,[9] we modified this method and optimised reaction conditions for a caucasian population.

The clinical use of these enzymes was examined in several selected patient groups, with particular attention being focused on those conditions which may, as a result of primary cardiorespiratory disturbance, lead to centrilobular necrosis. Alanine aminotransferase (ALT, EC 2.6.1.2) and gamma-glutamyltransferase activities were measured concurrently for comparative purposes.

Accepted for publication 4 March 1987

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Material and methods

GLUTAMATE DEHYDROGENASE

A Boehringer Mannheim kit, BM Test–Combination GLDH activated (Boehringer Mannheim, Mannheim, West Germany; catalogue number 124 320) was used in this study. The kit comprises three reagent phials; phial 1—triethanolamine buffer (50 mmol/l, pH 8·0) containing ammonium acetate and edetic acid at final concentrations of 0·1 mol/l and 2·5 mmol/l, respectively; phial 2—ADP and reduced nicotine adenine dinucleotide (NADH) at final concentrations of 1 mmol/l and 0·2 mmol/l, respectively; and phial 3 containing α-oxoglutarate, 7 mmol/l final concentration. The contents of phial 2 were dissolved in 1·5 ml of buffer from phial 1. Working reagent was then prepared by mixing 1 volume from phial 2 with 25 volumes from phial 1. Phial 3 containing substrate was added to the working reagent separately to start the reaction. GDH catalyses the conversion of oxoglutarate in the presence of ammonium ions to glutamate, with concomitant oxidation of NADH to nicotine adenine dinucleotide (NAD^+), which can be measured spectrophotometrically at 340 nm.

ALCOHOL DEHYDROGENASE

The assay for ADH was based on that described by Kato.[3] Reagents used were (i) glycine-NaOH buffer, 0·1 mol/l; (ii) NAD (Boehringer Mannheim, Mannheim, West Germany; catalogue No 127990, grade II, free acid, 5 g), 10 mmol/l in distilled water and prepared fresh each day; and (iii) ethanol, 20 mmol/l. Working reagent was prepared by adding 2 ml of NAD solution to 10 ml of buffer. Ethanol was added as a start reagent. ADH catalyses the conversion of ethanol to acetaldehyde with simultaneous reduction of NAD to NADH.

ALANINE AMINOTRANSFERASE

ALT was measured by the method recommended by the International Federation of Clinical Chemistry (IFCC)[10] (without activator) using a Roche Diagnostica kit (Roche Products Ltd, Dee Why, New South Wales, Australia) (catalogue No 071404) on the Cobas Bio centrifugal analyser (Roche Analytical Instruments, Nutley, New Jersey, United States of America).

GAMMA GLUTAMYLTRANSFERASE

GGT was also measured on the Cobas Bio by a Roche Diagnostica kit (catalogue No 07 1425 9) using the method of Szasz.[11]

Results

ADAPTATION OF GDH ASSAY TO COBAS BIO CENTRIFUGAL ANALYSER

Reaction kinetics

Reaction kinetics of the GDH assay were monitored for 900 seconds with the instrument settings shown in table 1. For patient samples with GDH activity of 100 U/l or less, the reaction was linear throughout the time interval measured.

Selection of optimised reaction conditions

In view of the observed linearity a minimum preincubation time of reagent of 10 seconds was considered to be suitable for the assay. To keep the total analysis time to roughly five minutes, an initial reading was taken 60 seconds after addition of sample and

Table 1 *Reaction conditions on Cobas Bio for measurement of GDH and ADH*

	Instrument settings	*GDH kinetic studies*	*GDH final assay conditions (20 µl)*	*ADH assay pH optimum studies*	*ADH kinetic studies*	*ADH final assay conditions*
1	Units	U/l	U/l	U/l	U/l	U/l
2	Calculation factor	1984	1984	1323	1323	1323
3	Standard 1 concentration	0	0	0	0	0
4	Standard 2 concentration	0	0	0	0	0
5	Standard 3 concentration	0	0	0	0	0
6	Limit	0	0·252	0	0	0·185
7	Temperature (°C)	37·0	37·0	37·0	37·0	37·0
8	Type of analysis	3	3	3	3	3
9	Wavelength (nm)	340	340	340	340	340
10	Sample volume (µl)	20	20	30	30	30
11	Diluent volume (µl)	30	30	40	40	40
12	Reagent volume (µl)	250	250	300	300	300
13	Incubation time (seconds)	10	10	300	10	120
14	Start reagent volume (µl)	10	10	10	10	10
15	Time of first reading (seconds)	0·5	60	20	0·5	0·5
16	Time intervals (seconds)	30	10	10	30	10
17	No of readings	30	24	20	30	18
18	Blanking mode	1	1	1	1	1
19	Printout mode	1	1	1	1	2

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the reaction was then monitored for a further 240 seconds. Printout mode 1 (assessment of the linear part of the reaction curve) was found to be superior to printout mode 2 (assessment of all points on the reaction curve by linear regression) for data reduction as mode 2 recorded several noise flags with patient material. Table 1 lists the final instrument settings for this assay system.

Precision studies

Three patients' sera with low, moderate, and raised GDH activity (8, 45, and 160 U/l, respectively) were selected for both intrarun and interrun precision studies of the assay. Samples to be analysed for interrun precision were stored at −70°C. Table 2 summarises the results and shows that both assays are precise, with coefficients of variation at all levels of 5·1% or less.

Linearity

Serial dilutions were prepared from a patient sample with GDH activity of 750 U/l and used to assess the linearity of each method. The assay was linear up to 500 U/l.

Reference range

Serum from 100 apparently healthy subjects, all of whom had normal ALT activities, was used to derive a reference range for GDH. Data were processed using non-parametric statistics. The reference range for GDH was calculated as 0–5 U/l.

Stability

A trial to assess GDH stability was conducted. Three samples with low (5 U/l), moderate (50 U/l), and high (150 U/l) GDH activity were stored at 4°C, −20°C, and −70°C for one week. All samples, independent of their activity, were stable over the week when stored at −70°C. Results obtained for storage at 4°C and −20°C were similar and showed that for the low sample GDH activity was stable for 48 hours, while for the moderate and high samples, on average, 5% activity was lost each day, that is, after one week, there was nearly a 40% reduction in initial activity.

MODIFICATION AND OPTIMISATION OF ADH ASSAY ON COBAS BIO

Determination of pH optimum

In a study of Japanese subjets Kato determined the pH optimum for ADH, using glycine-NaOH buffer, to be 9·0[3]; however, the author qualified this finding by stating that the pH optimum would need to be redetermined for caucasians.

Using the assay conditions of Kato (table 1) buffers with pH of 8·0, 9·0, 10·0, 10·5 and 11·0 were prepared. A selection of patient samples were analysed using this range of buffers to determine an approximate pH optimum. From this preliminary experiment, optimum ADH activity was observed at pH 10·0. As a result, further buffers with pH's of 9·5, 9·8, 10·0 and 10·5 were prepared for detailed investigation.

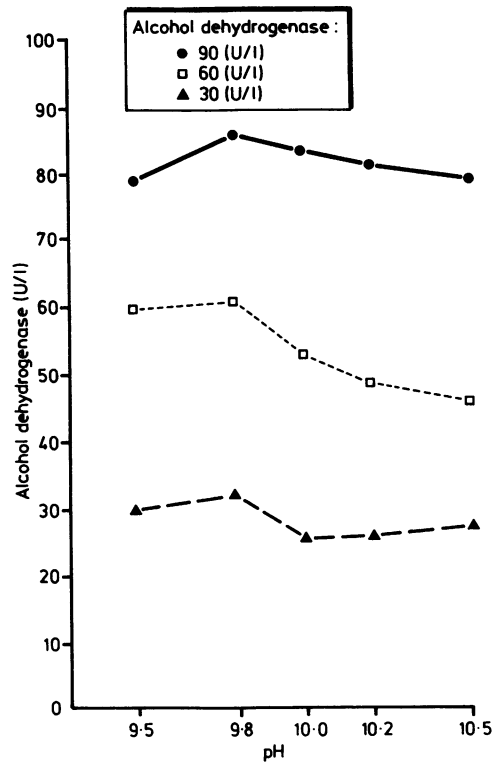

Fig 1 *Determination of pH optimum for ADH in caucasian population*

In each of four separate experiments three different patient samples, each with ADH activities of about 30, 60, and 90 U/l, were assayed with this range of buffers. Fig 1 shows representative data from one such experiment. While there was little difference in pH optimum between pH 9·5 and 9·8 at low ADH activity, it was clearly evident that the pH optimum for the caucasian population was 9·8 at higher enzyme activity.

Examination of reaction kinetics

The reaction kinetics of the ADH assay, using the instrument settings shown in table 1, were examined using a series of patient samples with ADH activities of 250, 150, or 35 U/l (fig 2). It was concluded that the five minute preincubation time recommended by Kato[3] could be reduced to 120 seconds. As substrate

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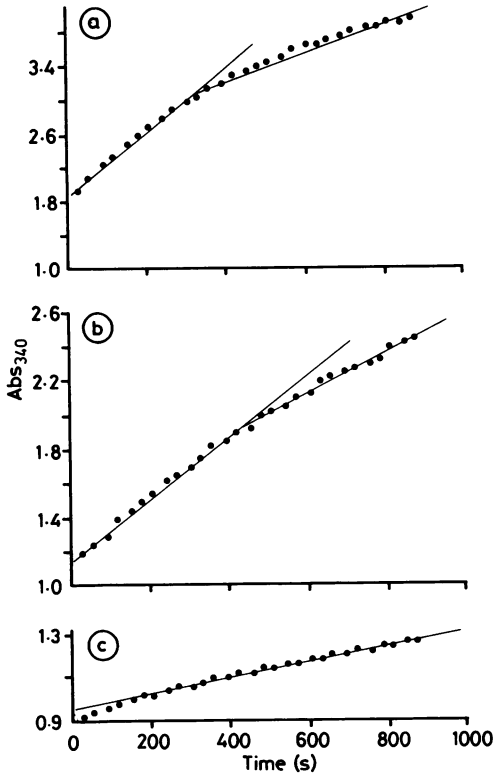

Fig 2 *Reaction kinetics of ADH assay. ADH activity in these samples was a) 250 U/l, b) 150 U/l, and c) 35 U/l.*

depletion occurred after 360 seconds with ADH samples greater than 250 U/l, a reading interval of 180 seconds (18 readings at 10 second intervals) was selected for our assay system. Total analysis time was therefore five minutes. Activities were calculated using linear regression (printout mode 2). Table 1 lists final instrument settings for the ADH assay.

Linearity

Two patient samples, with activities of 400 and 35 U/l, respectively, were each diluted serially in saline and analysed on the Cobas Bio. Non-linearity was observed with both samples and was more exaggerated in the dilution of the low sample. A similar finding has been reported by the authors[12] in studies with intestinal alkaline phosphatase, when a sample of high activity was diluted in saline. The two ADH samples were serially diluted in inactivated serum (prepared by heating serum at 65°C for 10 minutes). In contrast to the results found using saline as diluent, linearity was observed in both samples using the serum based diluent (fig 3). A repeat of the linearity experiment using a further patient sample (total activity 480 U/l) diluted with inactivated serum confirmed that the linear range of the ADH assay was 0–250 U/l.

Table 2 *Precision studies for GDH assay using patient sera with low, moderate, and high GDH activity*

	Low	*Moderate*	*High*
Intrarun precision:			
Mean	7·3	45·8	160·9
SD	0·15	0·59	1·20
CV%	2·0%	1·3%	0·7%
(n =)	10	10	10
Interrun precision:			
Mean	6·9	44·5	155·7
SD	0·27	0·30	1·25
CV%	3·9%	0·7%	0·8%
(n =)	10	10	10

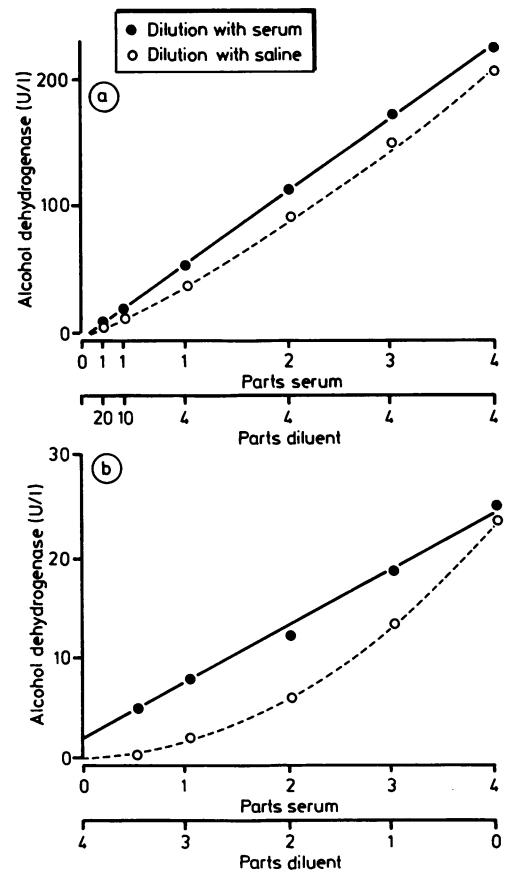

Fig 3 *Linearity studies: differences observed using saline and inactivated serum as diluent. ADH activity in these samples was a) 400 U/l, and b) 25 U/l.*

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Table 3 *Precision studies for ADH assay using patient sera with low, moderate, and high GDH activity*

	Low	Moderate	High
Intrarun precision:			
Mean	4·7	38·9	115·1
SD	0·45	0·40	0·71
CV%	1·0%	1·02%	0·6%
(n =)	10	10	10
Interrun precision:			
Mean	8·7	45·7	132·0
SD	0·35	1·35	1·82
CV%	4·0%	3·0%	1·4%
(n =)	10	10	10

Precision studies

Intrarun and interrun precision data were obtained using different patient sera with low, moderate, and raised activities of ADH. Table 3 summarises the results. Precision recorded using this assay system was superior to that documented by Kato.[3]

Reference range

From a population of 70 apparently healthy subjects, a reference range of 0–7 U/l for ADH was calculated using non-parametric statistics. This compares favourably with the range of 0–5 U/l determined by Kato.[3]

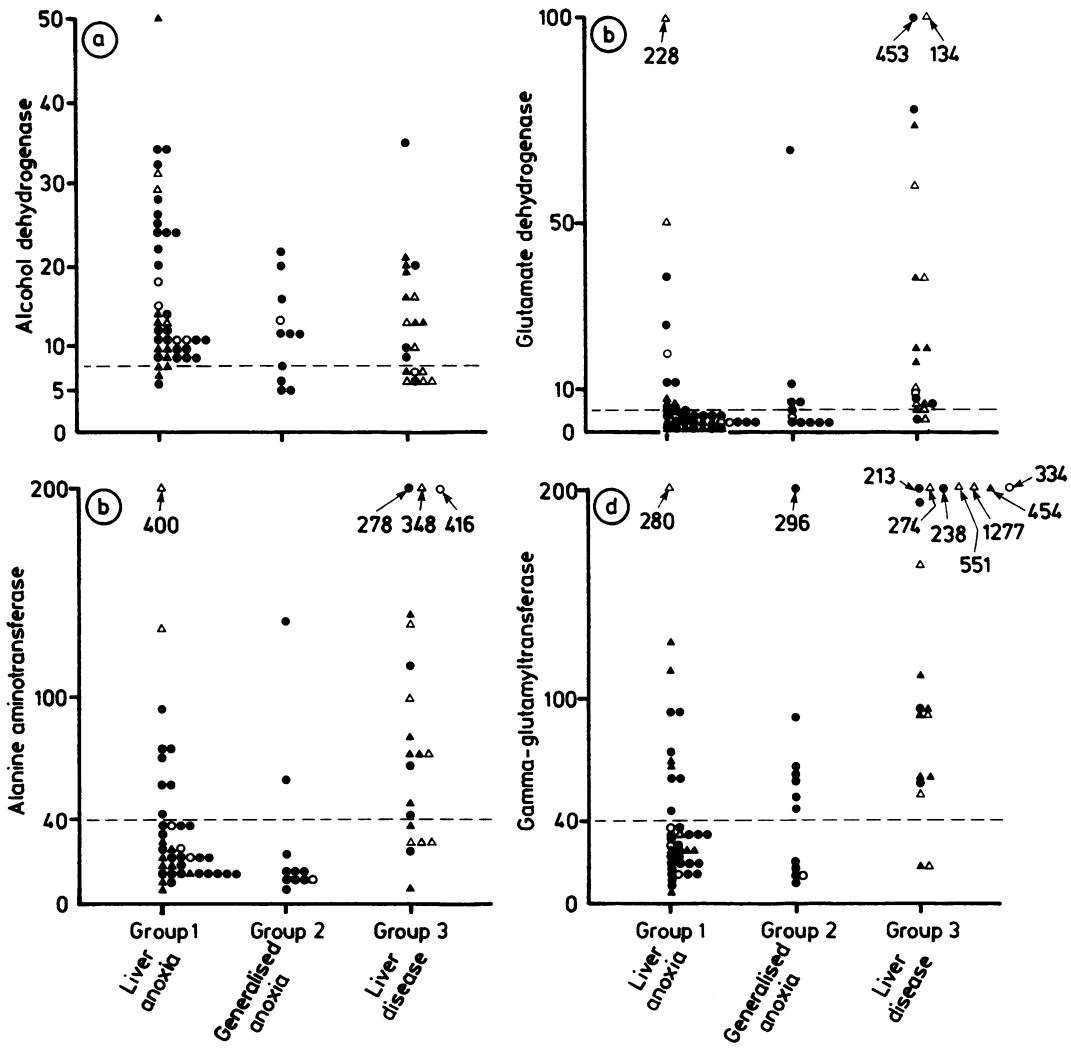

Fig 4 *Activities of ADH, GDH, ALT and GGT (U/l) found in selected patient groups. Group 1: liver anoxia (centrilobular damage) due to congestive cardiac failure (●), left ventricular failure (○), chronic obstructive airways disease (▲), or pulmonary oedema (△); Group 2: generalised anoxia, postoperatively (●), or due to trauma (○); Group 3: liver disease due to obstructive jaundice or cirrhosis (●), viral hepatitis (○), alcoholic liver disease (▲) or carcinoma (△).*

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Table 4 *Summary of clinical sensitivity of ADH, GDH, ALT, and GGT in specific diseases or groups*

Group/ subgroup	Disease	Clinical sensitivity (%)			
		ADH	*GDH*	*ALT*	*GGT*
1	Liver anoxia	90	25	25	25
1a	Congestive cardiac failure	96	16	28	24
2	Generalised anoxia	70	40	20	60
3	Liver disease	65	80	70	90
3a	Alcoholic liver disease	86	86	71	86
3b	Carcinoma	43	71	57	86

Stability

Stability of ADH was a major problem: when stored at either 4°C or −20°C, on average, 20% activity was lost within 24 hours of sample collection, followed by a further 10% loss of activity each subsequent 24 hours. In contrast, samples stored at −70°C remained completely stable for up to seven days.

Clinical evaluation

A detailed study was undertaken to assess the clinical use of ADH and GDH. Alcohol dehydrogenase, GDH, ALT, and GGT activities were measured on the plasma from over 200 patients with a variety of clinical disorders. From this initial group, 70 were selected for specific examination. Based on their primary clinical diagnosis, these patients were classified into one of three groups:

(i) *Group 1:* Liver anoxia due to centrilobular damage (n = 40). Patients in this category were diagnosed with the following cardiorespiratory disorders: congestive cardiac failure (n = 25), left ventricular failure (n = 5), chronic obstructive airways disease (n = 8), or pulmonary oedema (n = 2), all of which are known to cause liver anoxia and, potentially, centrilobular necrosis.

(ii) *Group 2:* Generalised anoxia (n = 10) following surgery or multiple trauma.

(iii) *Group 3:* Liver disease (n = 20). Patients in this group were diagnosed as having obstructive jaundice or cirrhosis (n = 5), viral hepatitis (n = 1), alcoholic liver disease (n = 7) and carcinoma (n = 7).

Fig 4 shows the activities of each enzyme found in these patients. The clinical sensitivity—that is, the number of abnormal test results in those patients with disease—of each enzyme for each group and selected subgroups (those with n > 5) was also documented (table 4).

Table 4 shows that ADH is a highly sensitive marker for liver anoxia and resultant centrilobular damage caused by cardiorespiratory disturbance (in particular congestive cardiac failure). This finding confirms and extends the preliminary work of Kato.[4] Our data show that ADH is also a sensitive indicator of generalised anoxia. In terms of specific liver disease, and in particular, carcinoma of the liver, GGT was found to be superior to the other enzymes tested. GDH does not seem to have a role in detecting liver anoxia, but is at least equivalent in sensitivity to ALT as a marker for liver disease. In many cases of liver disease, an increase in ALT activity was paralleled by an increase in GDH, but the relative increase in activity—that is, the number of times greater than the top of the reference range—was far greater with GDH than with ALT. All enzymes were equally sensitive in detecting alcoholic liver disease.

Conclusion

We have developed and adapted methods for the determination of serum alcohol dehydrogenase and glutamate dehydrogenase for use with a centrifugal analyser. These enzymes were shown in a clinical study to be relatively useful markers for detection of a variety of specific liver disease states, though they were not more clinically sensitive than conventional liver enzymes such as alanine aminotransferase and, particularly, gamma-glutamyltransferase. Our findings suggest, however, that alcohol dehydrogenase may have considerable diagnostic potential in the detection of generalised anoxia and, in particular, anoxic damage to the liver caused by cardiorespiratory disorders.

We thank Dr G White for his constructive comments during the preparation of this manuscript. We are grateful to the Drug and Alcohol Services Council for their generous support.

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